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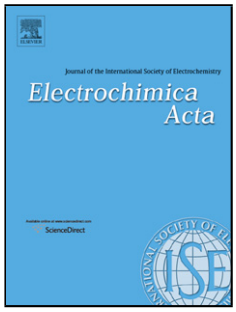
Published in:
Electrochimica Acta

Link to article, DOI:
10.1016/j.electacta.2017.08.126

Publication date:
2017

Document Version
Peer reviewed version



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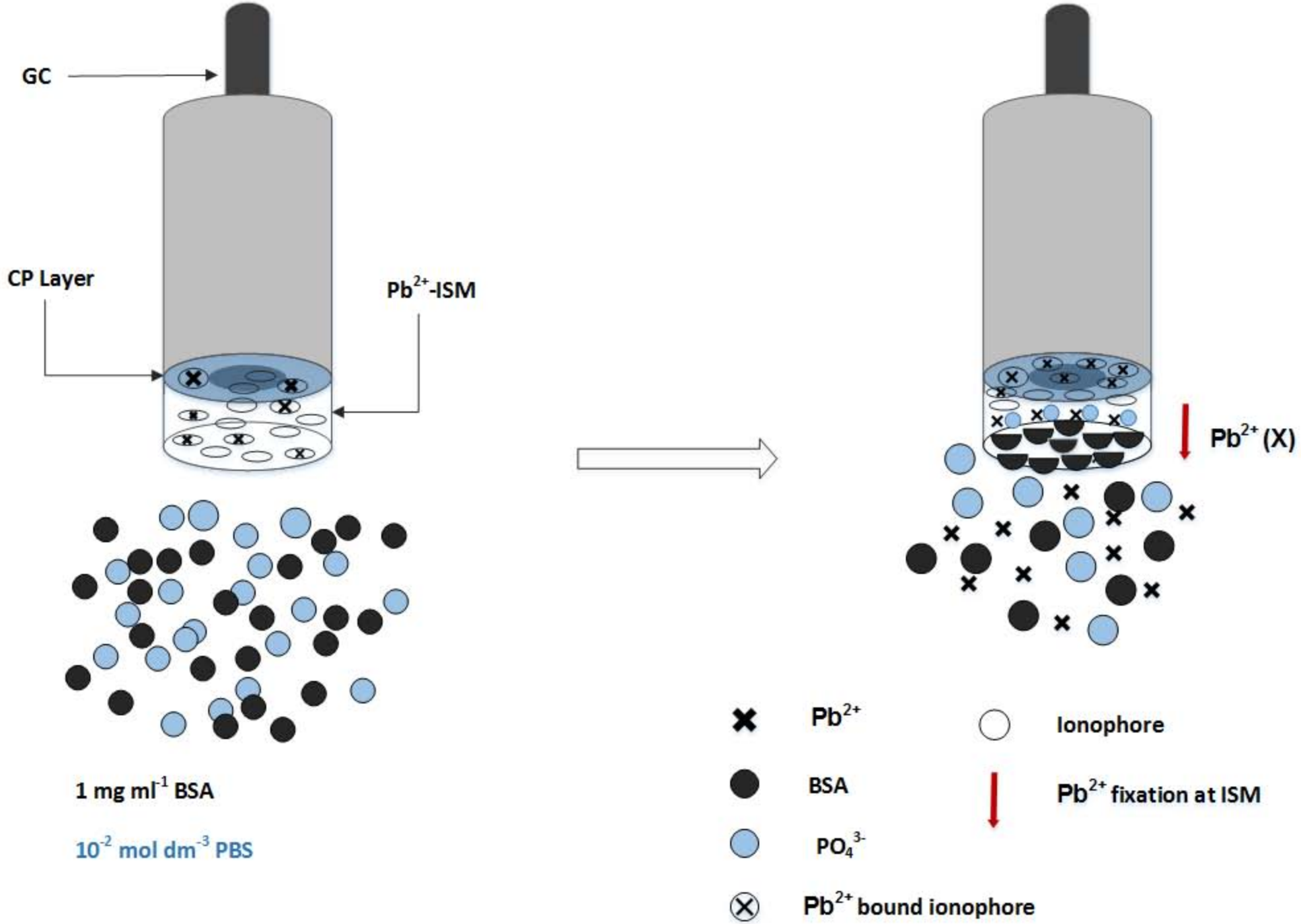
PII: S0013-4686(17)31780-2
DOI: http://dx.doi.org/doi:10.1016/j.electacta.2017.08.126
Reference: EA 30130

To appear in: *Electrochimica Acta*

Received date: 16-5-2017
Revised date: 19-8-2017
Accepted date: 22-8-2017

Please cite this article as: N.K. Joon, N. He, M. Wagner, M. Cárdenas, J. Bobacka, G. Lisak, Influence of phosphate buffer and proteins on the potentiometric response of a polymeric membrane-based solid-contact Pb(II) ion-selective electrode, *Electrochimica Acta* (2017), http://dx.doi.org/10.1016/j.electacta.2017.08.126


GC
CP Layer
Pb2+-ISM
1 mg ml-1 BSA
10-2 mol dm-3 PBS
Pb2+ (X)
Pb2+
BSA
PO43-
Pb2+ bound ionophore
Ionophore
Pb2+ fixation at ISM

Influence of phosphate buffer and proteins on the potentiometric response of a polymeric membrane-based solid-contact Pb(II) ion-selective electrode

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Highlights

- Influence of phosphate buffer and proteins on potentiometric response of Pb^{2+}-ISEs
- Lowering the detection limit of Pb^{2+}-ISEs
- Novel determination protocol of lead at low concentrations

Abstract

In this work, the influence of phosphate buffer and proteins on the potentiometric response of a polymeric membrane-based solid-contact Pb^{2+}-selective electrode (Pb^{2+}-ISE) was studied. The effects of bovine serum albumin (BSA) adsorption at the surface of the ion-selective membrane combined with electrode conditioning in phosphate-buffered saline (PBS) solution was elucidated by potentiometry and electrochemical impedance spectroscopy. The adsorbed BSA at the surface of the Pb^{2+}-ISE slightly lowered the detection limit but did not influence the selectivity of the Pb^{2+}-ISE towards the interfering ions studied (Cu^{2+}, Cd^{2+}). Conditioning of the Pb^{2+}-ISE in 0.01 mol dm^{-3} PBS resulted in a super-Nernstian response which was related to fixation/extraction of Pb^{2+} in the ion-selective membrane via precipitation of $Pb_3(PO_4)_2$ by PO_4^{3-} anions present in PBS. By conditioning of the Pb^{2+}-ISE in 0.01 mol dm^{-3} PBS + 1 mg/ml BSA it was possible to extend the linear response range of the Pb^{2+}-ISE towards lower analyte concentrations. The utilization of this conditioning procedure was validated by determination of Pb^{2+} concentrations down to ca 20 ppb in aqueous samples by Pb^{2+}-ISEs and by comparing the results with those obtained by ICP-MS.

Keywords: bovine serum albumin, phosphate-buffered saline, potentiometric sensors, non-equilibrium potentiometry, protein adsorption

1. Introduction

Potentiometric ion sensors (ion-selective electrodes, ISEs) are used routinely in clinical diagnostics for determination of electrolytes in biological fluids, e.g. bood plasma [1]. For the detection of biomolecules, potentiometric biosensors have been studied and used despite their greater complexity compared to ISEs [2-4]. In samples such as blood serum or urine, the sensors are exposed to a relatively complex sample matrix. Non-specific adsorption of e.g. blood plasma proteins and lipids at the sensor surface may lead to alteration of the sensor signal, leading to unreliable determination of the analyte [5-7]. The effect of such biofouling may be diminished by

deliberate immobilization of biomolecules at the surface of the sensor, prior to the actual determination. Irreversible adsorption of BSA at an ion-selective membrane was confirmed by means of in-situ potentiometry and ellipsometry. As the conclusion, it was suggested that a sensor must be equilibrated with the sample to avoid adsorption-related potential changes during the measurements [8]. However, introduction of biomolecules in potentiometric ion sensors may influence the analytical signal via e.g. metal binding by biomolecules such as proteins, which have been shown to influence the extent of released metals from various metal surfaces under biological conditions [9, 10]. Furthermore, ISEs have been used to study their interactions with proteins and the binding mechanism of halide ions to bovine serum albumin (BSA) and hemoglobin [11, 12].

The potentiometric response and protein adsorption at the ion-selective membrane (ISM) of ISEs have been previously studied [8, 13, 14]. The most commonly used method to immobilize biomolecules is via physisorption. In this process, electrostatic and Van der Waals interactions between the interface and the biomolecule lead to biomolecule adsorption at the liquid-solid interface [15]. For example, the isoelectric point of BSA is 4.7, which means that in phosphate-buffered saline (PBS at pH 7.4) the protein carries a negative charge. The negatively charged protein easily adsorbs on positively charged surfaces [8]. Another way to immobilize biomolecules at the interfaces is by covalent immobilization, where by chemical synthesis/modification the protein/biomolecule is chemically fixed onto the base material [13, 16]. A third immobilization procedure is electrochemical deposition. In this process, the immobilization may occur only on conducting surfaces (e.g. conducting polymers) by applying a positive or negative potential that will attract the biomolecules to the surface. Depending on the size of the biomolecules and the porosity of the electrode material, the biomolecules may deposit on the electrode surface and also enter into the bulk of the electrode material [17]. Additionally, a target biomolecule (template) may be immobilized at the interface and in the bulk of the material via chemical (or electrochemical) polymerization forming a polymer network with binding sites for a specific biomolecule (molecular imprinting) [14, 18, 19].

Introducing a metal complexing agent into the solid contact of the ISE or into the conditioning solution, has been used to extend and recover (maintain) the Nernstian response of the ISE at low analyte concentrations [19-21]. In one of the approaches, it was demonstrated that by conditioning of Pb^{2+}-ISEs in a solution containing EDTA as the complexing agent (EDTA

complexed Pb^{2+} released from the ion-selective membrane in the vicinity of the sensor surface) a novel analytical protocol for ion determination including recovery of the low detection limit was developed [20]. Such protocols, however, consisted only of a small fraction of the widely investigated research area devoted to pushing the lower detection limits of potentiometric ion sensors [22-28].

In this work, the synergistic effect of protein (BSA) adsorption on the Pb^{2+}-selective membrane together with extraction/fixation of Pb^{2+} ions from the Pb^{2+}-selective membrane by PBS (pH = 7.4) in the conditioning solution are investigated. The effects of BSA and PBS on Pb^{2+}-ISEs are compared with the effects of BSA and PBS on K^+-ISEs and Cl^--ISEs.

2. Materials and methods

2.1 Reagents

Lead ionophore IV, potassium tetrakis(4–chlorophenyl) borate (KTpClPB), potassium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (KTFPB), 2-Nitrophenyl octyl ether (*o*-NPOE), poly(vinyl chloride) high molecular weight (PVC), tetrahydrofuran (THF), tridodecylmethylammonium chloride (TDMACl), poly(sodium 4-styrenesulfonate (NaPSS), Mw ~70,000), 3,4-ethylenedioxythiophene (EDOT), phosphate-buffered saline (PBS, tablets) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Lead(II) nitrate ($Pb(NO_3)_2$) was purchased from Fluka. Aqueous solutions were prepared with freshly deionized water of 18.2 MΩ cm resistivity obtained with the ELGA purelab ultra water system. Phosphate-buffered saline (PBS) at 0.01 mol dm^{-3} was prepared by dissolving readymade tablets in deionized water to obtain 0.01 mol dm^{-3} Na_2HPO_4, 0.0018 mol dm^{-3} KH_2PO_4, 0.0027 mol dm^{-3} KCl and 0.137 mol dm^{-3} NaCl.

2.2 Electrode preparation

Glassy carbon (GC) electrodes in a PTFE body were firstly polished using 0.3 μm Al_2O_3 and rinsed vigorously with deionized water. An Autolab PGSTAT 30 (Metrohm) controlled by a General Purpose Electrochemical System (GPES) software was used for electrodeposition of PEDOT onto GC electrodes. PEDOT(PSS) (for cation selective ISEs) or PEDOT(Cl) (for anion selective ISEs) was electropolymerized in a three-electrode electrochemical cell containing a GC

disk electrode as working electrode (WE), GC rod as counter electrode (CE) and a single junction Ag/AgCl/3M KCl reference electrode (RE). The polymerization was performed according to the procedure described elsewhere [29]. After electropolymerization, deionized water was used to rinse the prepared GC/PEDOT(PSS) or GC/PEDOT(Cl) WEs, followed by 2 h drying in air. Then the ISM was cast on top of the electrodes from the membrane cocktail consisting of 200 mg dry mass dissolved in 2 ml of THF (for Pb^{2+}-ISEs: 1% lead(II) ionophore IV, 0.5 % KTpClPB, 65.2% PVC and 33.3% o-NPOE; for K^{+}-ISEs: 1% potassium ionophore I, 0.4% KTFPB, 65.7% DOS, 32.9% PVC; for Cl^{-}-ISEs: 15% TDMACl, 51% o-NPOE, 34% PVC, all in (w/w %)). The final volume of the cocktail applied on each electrode was 60 μl (applied in three portions of 20 μl each) followed by evaporation of THF (approx. 1 min between each addition). The resulting thickness of the ISM after the evaporation of THF was approx. 60 μm. Immobilization of BSA by physical adsorption at the surface of the ISM (as indicated below) was done by immersing the ISEs for 30 min. in a solution of 1 mg ml^{-1} BSA in 0.01 mol dm^{-3} PBS. This was followed by vigorous rinsing with deionized water in order to remove unbound or loosely bound BSA from the ISM. The concentration of 1 mg ml^{-1} BSA was chosen in order to reach full monolayer coverage of the protein on the surface of the ISM, which corresponds to approx. 2 mg m^2 BSA surface concentration [8, 15].

2.3 Potentiometry

Potentiometric measurements were performed for unconditioned and conditioned electrodes (as further indicated). Between measurements, the electrodes were stored either in a conditioning solution of 10^{-3} mol dm^{-3} $Pb(NO_3)_2$ or in 0.01 mol dm^{-3} PBS with/without 1 mg ml^{-1} BSA (as further indicated). Calibration of Pb^{2+}-ISEs was done preparing the standard solutions or by automatic dilution of a stock solution using two Metrohm Dosino 700 instruments equipped with burets of 50 ml capacity (Herisau, Switzerland). The pumps were programmed to dilute the sample solution with freshly deionized water (18.2 MΩ cm) every 5 minutes. A single junction Ag/AgCl/3M KCl was used as RE. All potentiometric measurements were carried out in a 100 ml glass cell. The electromotive force (*EMF*) was recorded with an EMF16 Interface (Lawson Labs Inc., Malvern, PA, USA). All experiments were performed at room temperature (23 ± 2 ^{o}C). The activity coefficients were calculated according to the Debye-Hückel approximation. All the *EMF* data were corrected for liquid-junction potentials according to the Henderson equation.

2.4 *Selectivity of Pb^{2+}-ISEs*

Potentiometric selectivity coefficients of unmodified and BSA-modified Pb^{2+}-ISEs, to Pb^{2+} over Cu^{2+} and Cd^{2+} were determined using Pb^{2+}-ISEs that were conditioned for 30 min in 0.01 mol dm^{-3} PBS without/with 1 mg ml^{-1} BSA. The conditioned electrodes were first exposed to interfering analytes ($Cd(NO_3)_2$ and $Cu(NO_3)_2$) and only then to $Pb(NO_3)_2$. The separate solution method was used to obtain apparent selectivity coefficients to Pb^{2+} over interfering ions. Calibration curves (from 10^{-1} to 10^{-3} mol dm^{-3}) were registered first in interfering ion solutions ($Cu(NO_3)_2$ and $Cd(NO_3)_2$) and then in the primary ion solution ($Pb(NO_3)_2$). The calibration curves were obtained as described above, by dilution of the 0.1 mol dm^{-3} stock solutions of interfering and primary ions. The selectivity measurements were performed three times and uncertainties were estimated.

2.5 *Electrochemical impedance spectroscopy (EIS)*

Impedance measurements were conducted with an Autolab PGSTAT 30 (Metrohm) controlled by the Frequency Response Analyzer (FRA) software. All measurements were made at room temperature (23 ± 2 ^{o}C) in a three-electrode, one-compartment electrochemical cell. The electrolyte was 0.01 mol dm^{-3} PBS, which stayed in contact with air during EIS measurements. The WE was a Pb^{2+}-ISE (GC/PEDOT(PSS)/ISM) that was modified or unmodified by BSA, and the CE was a GC rod. The RE was a single junction Ag/AgCl/3M KCl reference electrode. The EIS was made at dc-potentials equal to the open-circuit potential (OCP) in the frequency range 100 kHz–0.01 Hz (71 data points per measurement) by using an ac-excitation amplitude of 10 mV. The impedance data were fitted to equivalent electrical circuits by using the Nova 1.7 (Metrohm) software. The investigated electrodes were solid-contact Pb^{2+}-ISEs (GC/PEDOT(PSS)/ISM) that were conditioned as illustrated in Scheme 1.

2.6 *Determination of Pb concentration in synthetic and environmentally resembling samples*

Prior to the lead(II) determination the electrodes were used to investigate the influence of background electrolyte on the potentiometric responses of unmodified and BSA and PBS modified Pb^{2+}-ISEs at low analyte concentrations (between10^{-8} and 10^{-6} mol dm^{-3} $Pb(NO_3)_2$)

after electrodes pretreatment (conditioning all electrodes in 10^{-6} mol dm^{-3} $Pb(NO_3)_2$ for 24 h and subsequently modifying part of the electrodes with 0.01 mol dm^{-3} PBS solution containing 1 mg ml^{-1} BSA for 30 min). The potentiometric response was investigate in deionized water and in $10^{-3.2}$ mol dm^{-3} NaCl as background electrolyte. The potentiometric responses of unmodified and modified Pb^{2+}-ISEs were collected from 3 electrodes of each kind and uncertainties of potential traces and slopes were calculated.

Furthermore, Pb^{2+}-ISEs were used for the determination of Pb^{2+} concentration in synthetic and environmentally resembling samples. In all cases, a single-junction Ag/AgCl/3M KCl was used as reference electrode. The total Pb concentrations in the standards and samples were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer elan 6100 DRC plus). The environmentally resembling sample consisted of natural (river) water spiked with lead(II) at two different concentrations. Natural water (pH= 6.7) was collected from Aura River on 30th of June 2017 in Turku, Finland (60°27'54.0"N 22°18'25.0"E). The freshly collected river water was filtered with 1.0 µm pore size, Millipore membrane filter prior to preparation of standards and sample solutions. The natural water sample was analyze for its content using ICP-MS and was found to contain (major elements in random order, ppb): Fe (380), Mn (61), Ca (21788), K (3472), Al (297), Ba (25), Cu (4), Li (5), Mg (12161), Na (13350), Ni (4), Pb (0.8), Sr (103), Zn (79).

In the analysis of both sample types, firstly the two calibration standards of 10^{-6} mol dm^{-3} and 10^{-7} mol dm^{-3} of $Pb(NO_3)_2$ were prepared either in ultrapure water (synthetic samples) or natural water matrix (environmentally resembling samples). The total Pb concentrations in standards used for determination of lead in synthetic samples were found to be $10^{-6.1}$ mol dm^{-3} and $10^{-7.2}$ mol dm^{-3} lead while in standards used for determination in environmentally resembling samples were found to be $10^{-6.0}$ mol dm^{-3} and $10^{-7.1}$ mol dm^{-3} lead. In both cases, the concentrations obtained by ICP-MS were further used for preparation of the calibration curves. Two synthetic samples of $10^{-6.4}$ mol dm^{-3}, and $10^{-7.0}$ mol dm^{-3} were prepared by dilution of the previously prepared calibration solution ($10^{-6.1}$ mol dm^{-3} $Pb(NO_3)_2$) with ultrapure water (synthetic samples). Two environmentally resembling samples were prepared by addition of appropriate aliquots of concentrated lead(II) solution into the matrix of the natural water to result in $10^{-6.6}$ and $10^{-6.2}$ mol dm^{-3} $Pb(NO_3)_2$.

For the determination, one set of six Pb^{2+}-ISEs were conditioned in 10^{-7} mol dm^{-3} $Pb(NO_3)_2$ for 24 h (for the determination in the synthetic samples) and an other set of six Pb^{2+}-ISEs were conditioned in natural water solution containing 10^{-6} mol dm^{-3} $Pb(NO_3)_2$ for 24 h (for the determination in the environmentally resembling samples). Then three electrodes of each set of Pb^{2+}-ISEs were modified by conditioning for 30 min. in 0.01 mol dm^{-3} PBS solution containing 1 mg ml^{-1} BSA. Subsequently, each set of unmodified and modified Pb^{2+}-ISEs were used for the determination of the lead in the synthetic and environmentally resembling samples, respectively. In both cases, the *EMF* of the electrodes was measured firstly in solution containing lower and secondly in higher concentration of the primary $Pb(NO_3)_2$. As a result, a two-point calibration was obtained. Then, the measurements in synthetic and environmentally resembling samples were performed. The *EMF* was recorded for 5 (synthetic samples) and 1 (environmentally resembling samples) minutes in each calibration and sample solution. Multiple determinations of Pb^{2+} in synthetic samples were performed by repeating the procedure of calibration and measurement protocol (n = 6). The calibrations and the determinations of Pb were performed at room temperature (23 ± 2 ^{o}C).

3. Results and Discussion

3.1 Potentiometric response of ISEs with and without immobilized BSA

ISMs have been found to be biocompatible with biomolecules such as proteins, which can be defined as the ability of an ISM to interact with a biological surrounding without causing an adverse change to that surrounding, e.g. blood and plasma [6, 30]. Previously, it was also found that BSA forms a monolayer on top of a PVC-based ISM (K^+-ISM), changing to more hydrophilic the wettability of the membrane (BSA is more hydrophilic than the ISM) [8]. The adsorption of proteins at the ISM was found, however, to widely depend on the chemical composition of the ISM [31]. If BSA has a strong affinity towards the primary ion (containing multi-metal binding sites) [9], its presence on the ISM is expected to influence the potentiometric response of the sensor. Fig. 1 presents a comparison of the potentiometric response of Pb^{2+}-ISEs without (electrode **1**) and with (electrode **2**) adsorbed BSA. The response curves (Fig. 1) were recorded in standard solutions of $Pb(NO_3)_2$ after 24 h conditioning in 10^{-4} mol dm^{-3} Pb^{2+}. Importantly, BSA immobilization on electrode **2** was performed after conditioning of the electrode in 10^{-4} mol dm^{-3} Pb^{2+}. The calibration of all Pb^{2+}-ISEs was done first from 10^{-8} to 10^{-3}

mol dm^{-3} Pb^{2+} (filled symbols in Fig. 1) and then from 10^{-3} and 10^{-8} mol dm^{-3} Pb^{2+} (empty symbols in Fig. 1). All electrodes were characterized with close to Nernstian potentiometric response (27-29 mV dec^{-1}) between 10^{-3} to 10^{-6} mol dm^{-3} Pb^{2+} in calibrations performed by both decreasing and increasing the Pb^{2+} activity. The low detection limits (LDL) determined from calibrations performed by increasing the Pb^{2+} activity in the standard solutions were noticeably better ($10^{-6.4 \pm 0.1}$ and $10^{-7.2 \pm 0.1}$ for unmodified and BSA-modified Pb^{2+}-ISEs, respectively) than the ones where the LDL was determined from calibration performed by decreasing lead activity ($10^{-6.0 \pm 0.1}$ and $10^{-6.2 \pm 0.1}$ for unmodified and BSA modified Pb^{2+}-ISEs, respectively). Lowering of the detection limit of Pb^{2+}-ISEs was observed only when the BSA-modified electrodes were used in the calibration performed from low to high ion concentration. This suggests that the adsorbed BSA (and the PBS buffer) lower the detection limit of Pb^{2+}-ISEs, but this effect disappears after the electrodes have been in contact with solutions of higher Pb^{2+} concentrations. The adsorbed BSA at the ISM surface may bind Pb^{2+} from the standard solutions at low Pb^{2+} concentrations and limit the release of Pb^{2+} from the ISM, thereby slightly lowering the detection limit. However, the results indicate that the BSA-rich ISM surface becomes saturated with Pb^{2+} in the more concentrated standard solutions. The results obtained for three consecutive electrodes were found to be comparable for this type of sensor [32, 33].

Fig. 2 presents the dynamic potentiometric response of Pb^{2+}-ISEs in a solution of 0.01 mol dm^{-3} PBS with and without 1 mg ml^{-1} BSA. Pb^{2+}-ISEs that were well equilibrated in 0.01 mol dm^{-3} PBS (ΔEMF= 0.6 mV) were treated by the addition of BSA to result in a solution of 1 mg ml^{-1} BSA in 0.01 mol dm^{-3} PBS. This resulted in the initial adsorption of BSA at the surface of the Pb^{2+}-ISEs and a potential increase of about 9 mV that gradually returned to the initial potential value observed before addition of BSA. Subsequent repetition of the sequence resulted in a negative potential drift in pure 0.01 mol dm^{-3} PBS and a positive potential drift in 0.01 mol dm^{-3} PBS containing 1 mg ml^{-1} BSA. The potential changes come from the local equilibration of ISEs when in contact with a new solution matrix, which is typical for complex samples. Interestingly, previously it was found that the electronically conductive substrate used and the treatment of the electrodes have a significant contribution to conditioning/equilibration time of solid-contact ISEs based on PEDOT(PSS) and for glassy carbon-based electrodes the equilibration time was found to be between 5 to 13 min [34]. Furthermore, it was found that attachment of BSA at the ISM is irreversible [8]. Thus, the presence of the protein layer on the

ISM attached during the very first addition (deliberate adsorption of protein at the surface of ISEs) may diminish further attachment of the proteins from the solution. Negatively charged BSA at the ISM repels negatively charged BSA present in the solution [8], which in turn lowers the possibility of further adsorption of BSA and the related potential changes. Such behavior is described by so-called random sequential adsorption of proteins where, owing to the electrostatic repulsion of BSA molecules, the proteins do not block the entire surface of the sensors but are assumed to be uniformly distributed on the membrane and prevent further attachment of proteins on already protein-modified surfaces [35]. In this way, the transport of ions between the solution and the membrane is not affected by the adsorbed BSA but requires a local equilibration with a new heterogeneous (BSA-containing) surface. This behavior is vital considering practical applications of sensors in complex media such as blood plasma, whole blood and environmental samples [36, 37].

The selectivity of unmodified and BSA-modified Pb^{2+}-ISEs to Pb^{2+} over Cd^{2+} and Cu^{2+} was determined in order to find out if the adsorbed BSA at the surface of Pb^{2+}-ISEs influences the sensor selectivity. The slopes of Pb^{2+}-ISEs in $Pb(NO_3)_2$ solutions used for calculating apparent selectivity coefficients were 31.8 ± 1.3 and 31.0 ± 1.1 for unmodified and BSA-modified electrodes, respectively. Similarly, the slopes of Pb^{2+}-ISEs in $Cd(NO_3)_2$ solutions were 33.4 ± 0.9 and 33.7 ± 0.5 mV dec^{-1} and in $Cu(NO_3)_2$ solutions were 29.7 ± 1.6 and 24.4 ± 3.4 mV dec^{-1} for unmodified and BSA-modified electrodes, respectively. The selectivity coefficients for Pb^{2+} over Cu^{2+} were $logK_{Pb^{2+},Cu^{2+}} = -7.69 \pm 0.44$ and $logK_{Pb^{2+},Cu^{2+}} = -7.31 \pm 0.24$ for unmodified and BSA-modified electrodes, respectively. Similarly, the selectivity coefficients for Pb^{2+} over Cd^{2+} were $logK_{Pb^{2+},Cd^{2+}} = -4.98 \pm 0.34$ and $logK_{Pb^{2+},Cd^{2+}} = -5.19 \pm 0.29$ for unmodified and BSA-modified electrodes, respectively. The obtained result indicates that the BSA present at the surface of the Pb^{2+}-ISEs does not influence the selectivity of the sensors.

3.2 EIS analysis

Pb^{2+}-ISEs were conditioned in PBS, BSA and $Pb(NO_3)_2$ solutions according to the protocol shown in Scheme 1 and thereafter studied by EIS. Examples of impedance spectra for the Pb^{2+}-ISEs are presented in Figs. 3 and 4 together with the equivalent circuits used to fit the EIS data. The model presented in Fig. 3 consists of a resistor ($R_{(RC)}$) and capacitor ($C_{(RC)}$) connected in parallel, followed by a Warburg impedance connected in series. For the second model (Fig. 4) the

above elements are included and combined in a Randles type equivalent circuit. This second model is used for electrodes that have been in contact with Pb^{2+} cations, where the additional resistor ($R_{(Rand.)}$) and capacitor ($C_{(Rand.)}$) are related to ion transfer at the ISM | electrolyte interface. The fit of the experimental data to these two models was acceptable (i.e. relatively low χ^2 values) as summarized in Table 1.

Based on the analysis of EIS data, $C_{(RC)}$ can be attributed to the geometric capacitance of the ISM, because the obtained values of $C_{(RC)}$ for all the studied electrodes are in the 10^{-11} F range. $R_{(RC)}$ can be interpreted as the bulk resistance of the ISM, while the admittance (Y_0) is related to Warburg diffusion in the ISM. As can be seen in Table 1, conditioning of the ISEs in 0.01 mol dm^{-3} PBS solution with/without 1 mg ml^{-1} BSA has only a minor influence on the impedance response of the ISEs (when taking into account the experimental uncertainties). Thus, protein adsorption does not significantly influence the charge transfer processes in the Pb^{2+}-ISE. On the other hand, conditioning in 10^{-3} mol dm^{-3} $Pb(NO_3)_2$ solution results in an additional interfacial charge-transfer process modelled by $R_{(Rand.)}$ and $C_{(Rand.)}$. Furthermore, ISEs that have been in contact with Pb^{2+} show a decrease in Y_0, which can be related to a lower diffusion coefficient of Pb^{2+}-ionophore complexes compared to diffusion of free ions (present in PBS) in the ISM. It should be emphasized that the EIS measurements were made in pure 0.01 mol dm^{-3} PBS (without Pb^{2+}) so the additional interfacial impedance ($R_{(Rand.)}$ and $C_{(Rand.)}$) must be related to interactions between PBS in the solution phase with Pb^{2+} ions that accumulated in the ISM during conditioning. This effect is studied in more detail below for Pb^{2+}-ISEs in comparison with K^+-ISEs and Cl^--ISEs.

3.4 Effect of conditioning of ISEs in 0.01 mol dm^{-3} *PBS and 1 mg* ml^{-1} *BSA solution on the potentiometric response*

Conditioning the Pb^{2+}-ISEs in a solution containing BSA and PBS was performed to evaluate the effects of adsorbed BSA at the Pb^{2+}-ISM | solutions interface in combination with the effect of PO_4^{3-} present in PBS. For this purpose, different conditioning times of Pb^{2+}-ISEs in 0.01 mol dm^{-3} PBS with or without 1 mg ml^{-1} BSA were used. Figs. 5 A and B present the calibration curves of Pb^{2+}-ISEs between 10^{-8} and 10^{-3} mol dm^{-3} Pb^{2+} after different conditioning time in 0.01 mol dm^{-3} PBS with 1 mg ml^{-1} BSA (Fig. 5 A) and in 0.01 mol dm^{-3} PBS without BSA (Fig. 5 B). The results were compared to the potentiometric response of unconditioned Pb^{2+}-ISEs, where a

super-Nernstian response between 10^{-4} and 10^{-6} mol dm^{-3} Pb^{2+} was observed. This is the typical behavior observed for ISEs that have not been conditioned with the primary ion. The super-Nernstian response is related to the flux of Pb^{2+} ions into the ISM due to binding of Pb^{2+} by the ionophore in the membrane, resulting in depletion of Pb^{2+} in the solution layer close to the membrane (at low concentrations of Pb^{2+} in solution) [38-40]. By conditioning in primary ion, the primary ion is introduced to the membrane and the ion uptake effect was not anymore visible as the ISM becomes equilibrated in terms of a constant Pb^{2+} concentration inside of the membrane. Thus, the Pb^{2+}-ISE conditioned in primary ion showed the typical low detection limit of approx. $10^{-6.5}$ mol dm^{-3} Pb^{2+} [33]. Further (0.5 h) conditioning of the Pb^{2+}-ISE in 0.01 mol dm^{-3} PBS with 1 mg ml^{-1} BSA lowered the detection limit to $10^{-7.5}$ mol dm^{-3} Pb^{2+}, while longer conditioning (3 and 6 h) again gave a super-Nernstian response (Fig. 5 A). The same trend was observed after prolonged conditioning of the Pb^{2+}-ISE in 0.01 mol dm^{-3} PBS (without BSA) (Fig. 5 B). However, the effect of PBS+BSA (Fig. 5 A) is more pronounced than that of PBS alone (Fig. 5 B), indicating a synergistic effect from PBS and BSA.

When considering the solubility product of $Pb_3(PO_4)_2$ ($S = 10^{-40.5}$) and the solution pH (pH 7.4) it can be estimated that 0.01 mol dm^{-3} PBS contains enough PO_4^{3-} to cause precipitation of Pb^{2+} in the form of $Pb_3(PO_4)_2$ at Pb^{2+} concentrations higher than ca. 10^{-9} mol dm^{-3} [41]. Precipitation of $Pb_3(PO_4)_2$ at the Pb^{2+}-ISM | solution interface can therefore not be excluded when the electrode is in contact with in 0.01 mol dm^{-3} PBS (with/without BSA). Such precipitation may result in depletion of Pb^{2+} at the Pb^{2+}-ISM | solution interface and extraction of Pb^{2+} from the ISM, leading to the occurrence of the super-Nernstian slopes observed in the potentiometric measurements (Figs. 5A and 5B). This finding is principally different from the previously investigated application of solution based methods to renew the low detection limit by application of Na_2EDTA conditioning solution in order to extract Pb^{2+} from the ISM into the solution phase [20]. To confirm this hypothesis, the influence of PBS on the potentiometric response for ISEs (where no precipitation of the primary ion occurs due to PBS) was evaluated by using the same measurement protocol. The results obtained for K^+-ISEs and Cl^--ISEs are shown in Figs. 5C and 5D, respectively. The potentiometric response for K^+-ISEs and Cl^--ISEs were not influenced by conditioning in 0.01 mol dm^{-3} PBS with 1 mg ml^{-1} BSA, which indicates the lack of formation of PO_4^{3-} based salts in these ISMs.

The influence of the BSA concentration on the extraction of Pb^{2+} from the Pb^{2+}-ISM were studied by applying different BSA concentrations (0, 0.1, 0.25, 0.5, 1 and 2 mg ml^{-1} BSA) in 0.01 mol dm^{-3} PBS for 3 h and recording the calibration curves from low to high (10^{-8} and 10^{-3}) and subsequently from high to low (10^{-3} and 10^{-8}) mol dm^{-3} Pb^{2+}. The results are shown in Fig. 6 for Pb^{2+}-ISEs that were initially conditioned in 10^{-3} mol dm^{-3} Pb^{2+} for 24 h. The detection limit of the equilibrated Pb^{2+}-ISEs was approx. $10^{-6.5}$ mol dm^{-3} Pb^{2+}, no matter the direction of increasing or decreasing lead(II) concentration in the calibration sequence. Subsequently, such sensors were conditioned in 0.01 mol dm^{-3} PBS for 3 h and the high ion uptake effect (super-Nernstian response) was observed between 10^{-5} and 10^{-6} mol dm^{-3} Pb^{2+} during the calibration performed from low to high and high to low lead(II) activity. Once again, this confirms that the 0.01 mol dm^{-3} PBS acts as a complexing/precipitating agent for Pb^{2+} accumulated in the ISM during previous conditioning of the sensors. However, even the smallest addition of BSA to the conditioning solution influenced the sensor response. In all cases where BSA was added to the conditioning solution the response of every Pb^{2+}-ISE during the calibration performed from high to low Pb^{2+} activity resulted in an extended linearity of the calibration curve and improvement of the detection limit of the sensors (approx. $10^{-7.5}$ mol dm^{-3} Pb^{2+}). These results indicate that adsorbed BSA moderates the transport of Pb^{2+} at the Pb^{2+}-ISM | solution interface, which extends the linear response towards lower Pb^{2+} activities without causing any super-Nernstian response (Fig. 6).

The observed effects are transient, which means that the conditioning of the sensors in PBS buffer to precipitate Pb^{2+} accumulated in the ISM and modifying the sensors surface with BSA is time-limited and only with benefit of its applicability in measurements at low analyte concentrations. Previously it was shown that ion fluxes in ion-selective membranes are a key factor for the stability of the sensor response and the detection limits of ISEs [42, 43]. Thus by controlling the ion fluxes, lowering of the detection limit is possible. In such methods, the protocol of the measurement is very strict in terms of conditioning and measurement times and concentrations of the conditioning solutions. It relies on partial depletion of the primary ion in the membrane and its outer surface, which induces fluxes of primary ion towards the membrane phase. Despite using PBS to precipitate Pb^{2+} accumulated in the ISM, immobilizing BSA at the surface brings additional benefits. Namely, proteins adsorbed on the surface of PVC have charge and cation buffering capacity, which means that carrying negative charge they will attract cations

(Pb^{2+} in this work) present in the solution and this is why at low analyte concentration the elongation of the calibration curve for lead(II) is observed (Fig. 6.). Additionally, the BSA is taking part in the transport of ions from the solution to the membrane but at some point of time, saturation of BSA with cations is inevitable and re-immobilization of BSA is required (time-limited measurements). For these reasons the fate of the BSA at the surface, e.g. its lifetime associated with the possible denaturation of BSA is not a major concern in this specific analytical application.

3.5 Determination of Pb concentration in synthetic and environmentally resembling samples.

The influence of background electrolyte on the potentiometric response at low analyte concentrations of unmodified and BSA and PBS modified Pb^{2+}-ISEs is shown in Fig. 7. Owning to the developed PBS time-conditioning and BSA modification protocol (described in previous section) the slopes of calibration curves between 10^{-8} and 10^{-6} mol dm^{-3} Pb^{2+} were significantly better than for unmodified Pb^{2+}-ISEs. On the other hand the standard deviation of the standard potential between electrodes (three electrodes of each kind) were better for unmodified electrodes (between 1.5 and 2.6 mV) than for PBS and BSA modified electrodes (between 3.7 and 7 mV). The higher standard deviation of the potential traces came from the fact the electrodes operate on transient effects induced by strict concentration and time conditioning and modification while unmodified sensors are well equilibrated and close to their detection limits. In both cases, higher slopes were obtained in deionized water compared to the ones obtained in measurements performed with constant $10^{-3.2}$ mol dm^{-3} NaCl (concentration similar to the one usually observed in natural waters). Thus, slopes for unmodified Pb^{2+}-ISEs were 18.2 ± 0.5 and 14.6 ± 0.5 while for PBS and BSA modified Pb^{2+}-ISEs were 31.7 ± 1.1 and 28.0 ± 1.6 mV dec^{-1} for measurements done in deionized water and $10^{-3.2}$ mol dm^{-3} NaCl, respectively. The decrease of potentiometric response and change of the standard potential of unmodified Pb^{2+}-ISEs was slightly greater than for PBS and BSA modified Pb^{2+}-ISEs confirming that BSA moderates the transport of Pb^{2+} at the Pb^{2+}-ISM | solution interface (as described in previous section). Moreover, the selectivity of the Pb^{2+}-ISEs (based on lead ionophore IV) was good enough to discriminate the possible interfering sodium present in the standard solutions, e.g. $logk^{pot}_{Pb^{2+},Na^{+}} = -5.4$ [44].

Taking into account the Pb^{2+}-ISEs can discriminate high background of interfering ions (g. $logk^{pot}_{Pb^{2+},Na^{+}} = -5.4$, $logk^{pot}_{Pb^{2+},K^{+}} = -5.6$, $logk^{pot}_{Pb^{2+},Ca^{2+}} = -6.0$, $logk^{pot}_{Pb^{2+},Mg^{2+}} = -5.1$ [44]), electrodes were used for the determination of Pb concentration in synthetic and environmentally resembling samples. The synthetic samples contained 89.2 and 21.7 ppb Pb while environmentally resembling samples contained 53.3 and 136.7 ppb Pb (determined by ICP-MS). Two sets of six Pb^{2+}-ISEs (three unmodified and three modified) were prepared and calibrated as described in the experimental section (chapter 2.6). For synthetic samples determination, the slope for the calibration curve obtained using unmodified Pb^{2+}-ISEs was only 14.5 ± 0.6 mV, while the slope for the modified Pb^{2+}-ISEs was 27.5 ± 1.5 mV. For analysis of environmentally resembling samples, the slope for the calibration curve obtained using unmodified Pb^{2+}-ISEs was always below 10 mV (that did not result in any successful determination), while the slope for the modified Pb^{2+}-ISEs was 18.7 ± 2.4 mV. Significant decrease in the slopes of both kinds of electrode was caused by high concentration of various interfering ions and a high ionic strength of the standard and sample solutions. Table 2 presents the Pb concentration determined in synthetic 1 and 2 samples and environmentally resembling 3 and 4 samples using unmodified and modified Pb^{2+}-ISEs. The results were compared to those obtained by ICP-MS. The lack of linearity of the unmodified Pb^{2+}-ISEs at this low concentration range translated into higher errors of Pb determination (synthetic samples) or unsuccessful determination (environmentally resembling samples). In both sample types, the concentration of Pb determined using modified Pb^{2+}-ISEs was found to be comparable to the concentrations obtained by ICP-MS. In case of synthetic sample lead(II) determination, the repeatability and reproducibility of the determinations by Pb^{2+}-ISEs was studied by performing multiple Pb determination using unmodified and modified Pb^{2+}-ISEs. As can be seen in Table 2, the Pb^{2+}-ISEs that were modified by BSA showed a higher precision compared to the unmodified Pb^{2+}-ISEs. The results shows that the modification of Pb^{2+}-ISEs with BSA and PBS results in a lower detection limit of the sensors and allows more reliable determination of ion concentration in various sample types, e.g. environmental samples.

4. Conclusions

Immobilization of BSA at the outer surface of the ion-selective membrane (ISM | solution interface) of solid-contact Pb^{2+}-ISEs was successfully accomplished via simple and fast

physisorption. Conditioning of the Pb^{2+}-ISEs in a solution consisting of 0.01 mol dm^{-3} PBS and 1 mg ml^{-1} BSA extended the linear response range of the sensor towards lower analyte concentrations. This is related to a synergistic effect of Pb^{2+} complexation by BSA and extraction (precipitation) of Pb^{2+} from the ISM by PO_4^{3-} present in the PBS buffer solution. This conditioning procedure allowed accurate determination of Pb concentrations down to ca. 20 ppb. This work shows that a conditioning solution containing an ion that forms a sparingly soluble salt with the primary ion of the ISE, together with an adsorbed biomolecule at the surface of the ion-selective membrane, can be result in ISEs with improved analytical performance at low analyte concentrations. The co-application of PBS and BSA as conditioning solution is an interesting approach to solve environmental analytical problems related to e.g. determination of low concentrations of Pb(II).

ACKNOWLEDGEMENTS

GL and NKJ acknowledge financial support from the Rector of Åbo Akademi University via Åbo Akademis Jubileumsfond 1968- forskningssamarbete till Sverige as well as funding from the Academy of Finland (project number 295019).

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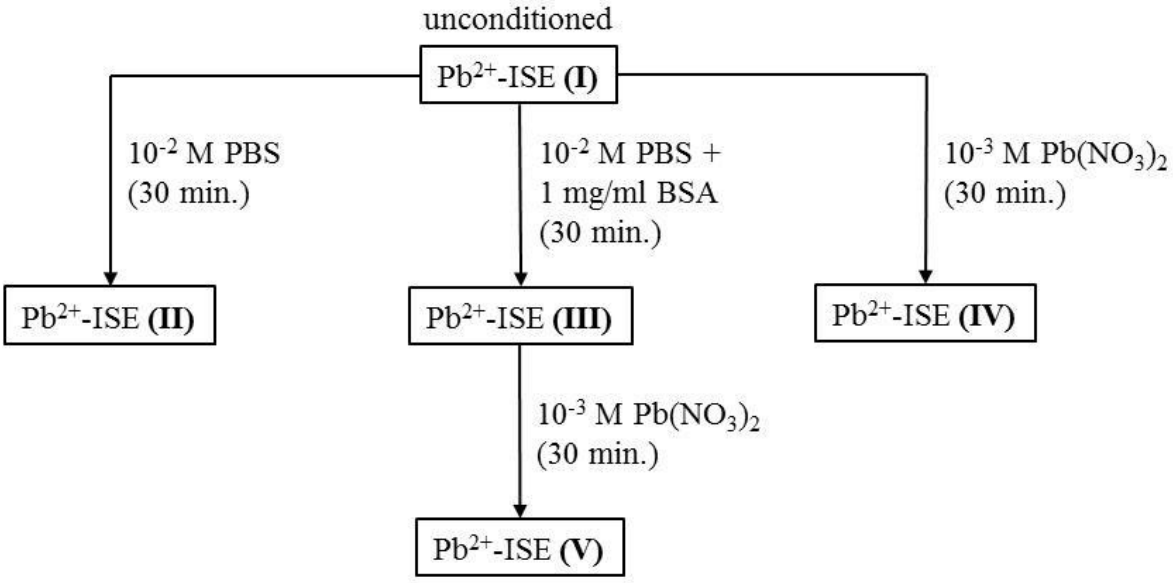

Scheme 1. Conditioning protocol for Pb^{2+}-ISEs **(I) – (V)**.

Table 1. Numerical values of model parameters obtained by fitting impedance data to the equivalent circuits shown in Figs. 3 and 4.

Electrode [a]	$R_{(RC)}$ / kΩ ($R_{(Rand.)}$ / kΩ) [b]	$C_{(RC)}$ / pF ($C_{(Rand.)}$ / μF) [b]	Y_0 / S $s^{0.5}$	χ^2
Pb^{2+}-ISE**(I)**	**81.2** ± 14.7	**27.3** ± 3.6	**3.68x10^{-5}** ± 3.31x10^{-6}	**0.040** ± 0.003
Pb^{2+}-ISE**(II)**	**86.0** ± 17.4	**25.9** ± 3.0	**3.60x10^{-5}** ±8.53x10^{-7}	**0.045** ± 0.003
Pb^{2+}-ISE**(III)**	**78.5** ± 7.3	**29.5** ± 5.5	**2.37x10^{-5}** ± 6.35x10^{-6}	**0.044** ± 0.030
Pb^{2+}-ISE**(IV)**	**106** ± 22.6 (**19.0** ± 3.5) [b]	**25.3** ± 3.4 (**0.636** ± 0.138) [b]	**1.36x10^{-5}** ± 1.55x10^{-6}	**0.035** ± 0.006
Pb^{2+}-ISE**(V)**	**86.9** ± 12.9 (**33.4** ± 14.9)[b]	**30.6** ± 5.9 (**0.389** ± 0.064)[b]	**1.73x10^{-5}** ± 1.60x10^{-6}	**0.127** ± 0.137

[a] Pb^{2+}-ISEs conditioned as described in Scheme 1.

[b] Values for $R_{(Rand.)}$ and $C_{(Rand.)}$.

Table *2.* Pb concentration (ppb) determined in synthetic (1 and 2) and environmentally resembling (3 and 4) samples.

Sensor / detection method	Sample 1	Sample 2
unmodified Pb^{2+}-ISEs*	76.6 ± 7.5	24.7 ± 3.3
unmodified Pb^{2+}-ISEs $^{\phi}$	79.1 ± 9.3	24.3 ± 3.6
BSA modified Pb^{2+}-ISEs*	87.3 ± 3.4	19.0 ± 1.9
BSA modified Pb^{2+}-ISEs $^{\phi}$	83.1 ± 4.7	18.2 ± 1.7
ICP-MS	89.2 ± 1.2	21.7 ± 0.5
Sensor / detection method	Sample 3	Sample 4
unmodified Pb^{2+}-ISEs $^{\phi}$	unsuccessful	unsuccessful
BSA modified Pb^{2+}-ISEs $^{\phi}$	51.1 ± 2.3	134.6 ± 8.7
ICP-MS	53.3 ± 0.7	136.7 ± 3.2

*measurement performed with a single (unmodified or modified) Pb^{2+}-ISEs (n=3)

$^{\phi}$ measurement performed with three different (unmodified or modified) Pb^{2+}-ISEs (n=6)

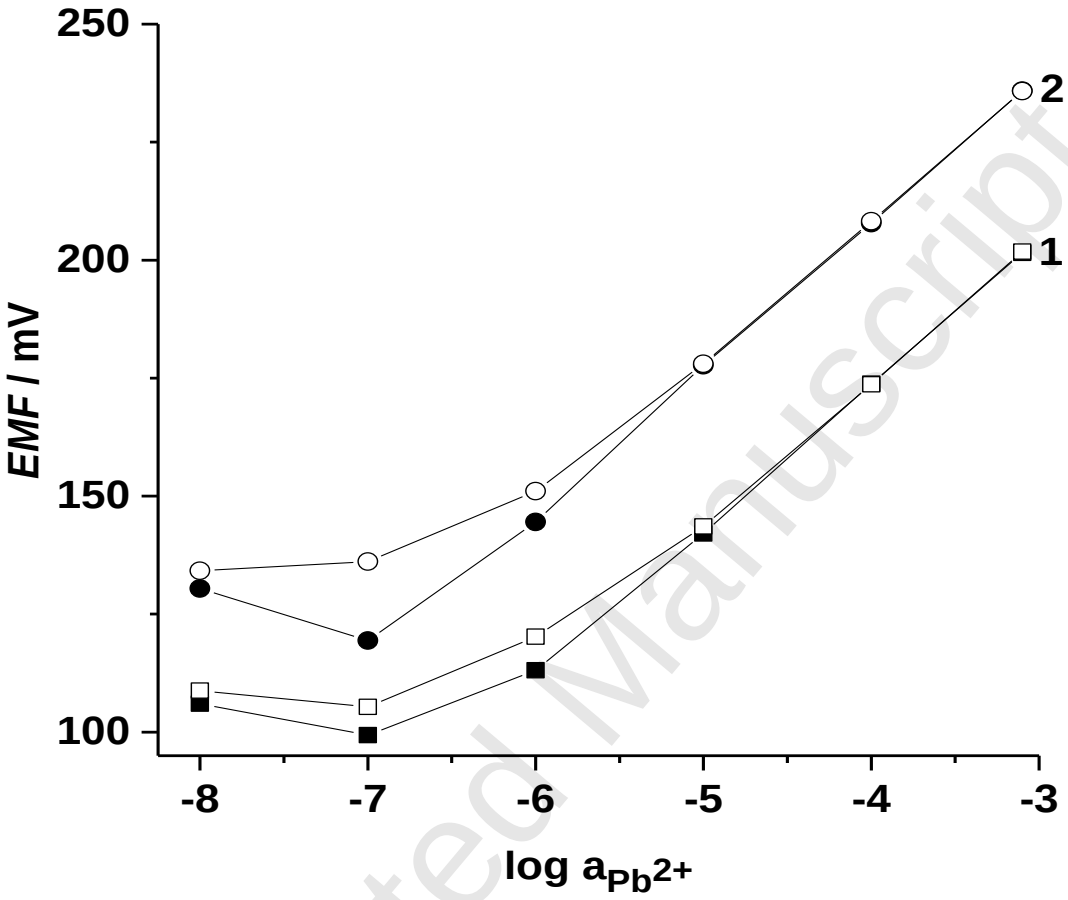

Fig. 1. Potentiometric response of unmodified Pb^{2+}-ISE (**1**) and Pb^{2+}-ISE modified with BSA and PBS (**2**) in standard solutions of $Pb(NO_3)_2$ after 24 h conditioning in 10^{-4} mol dm^{-3} Pb^{2+}. Calibrations were performed first from 10^{-8} to 10^{-3} mol dm^{-3} Pb^{2+} (filled symbols) and then from 10^{-3} to 10^{-8} mol dm^{-3} Pb^{2+} (empty symbols).

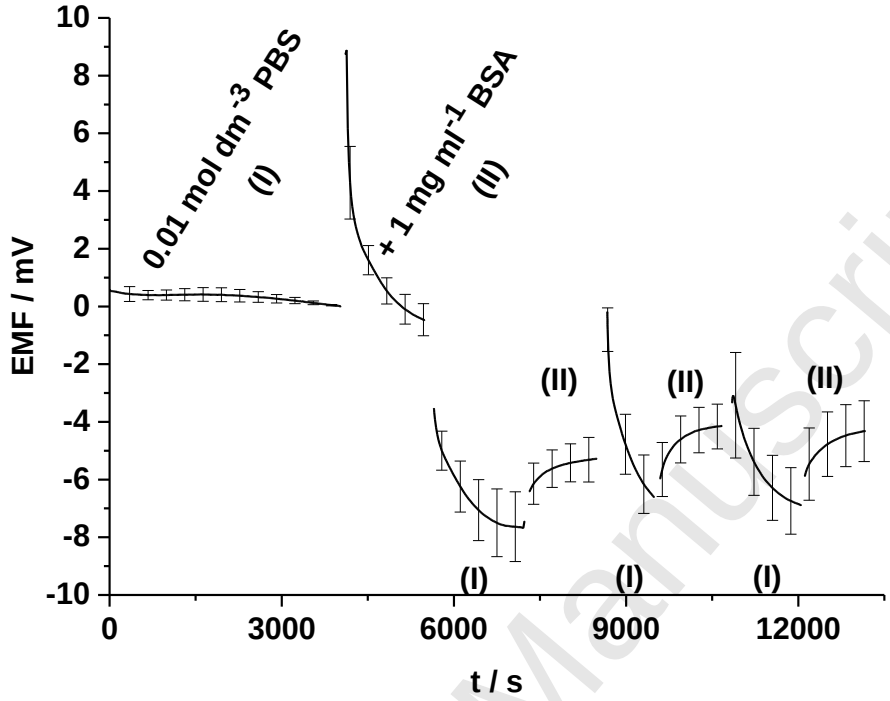

Fig. 2. Potential stability of Pb^{2+}-ISEs in 10^{-2} mol dm^{-3} PBS (**I**) after consecutive addition of 1 mg ml^{-1} BSA (**II**), repeated four times.

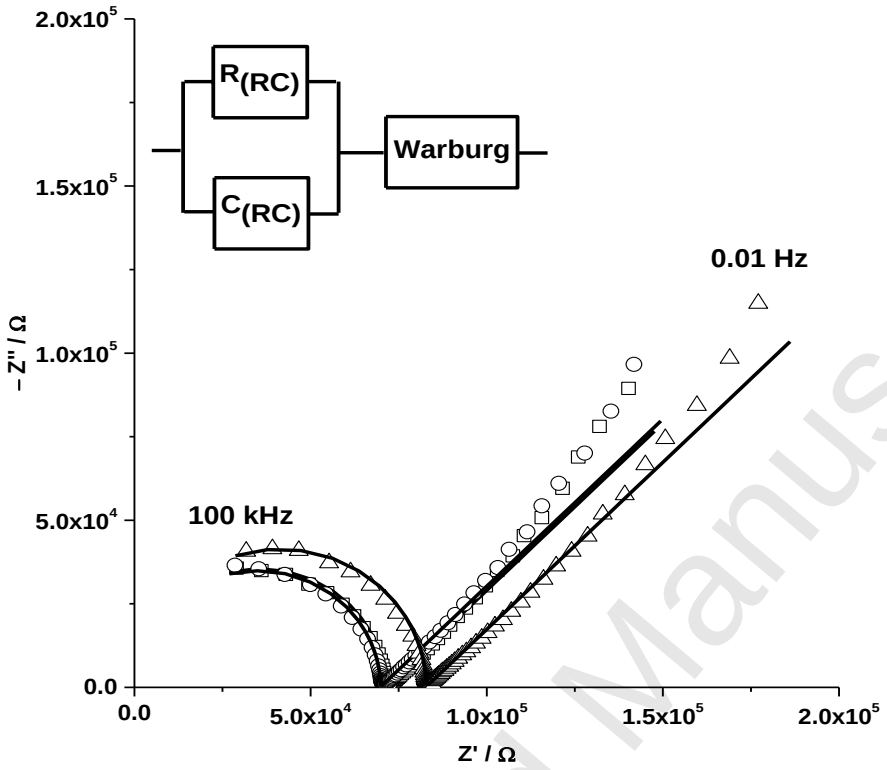

Fig. 3. Nyquist plots representing selected typical impedance response of the studied solid-contact Pb^{2+}-ISEs together with the fit (solid lines), where □ is an unconditioned Pb^{2+}-ISE (**I**), ○ is a Pb^{2+}-ISE conditioned in PBS (**II**) and Δ is a Pb^{2+}-ISE electrode conditioned in PBS and BSA (**III**). The numerals (**I**)-(**III**) refer to **Scheme 1**. Insert: equivalent circuit used for fitting.

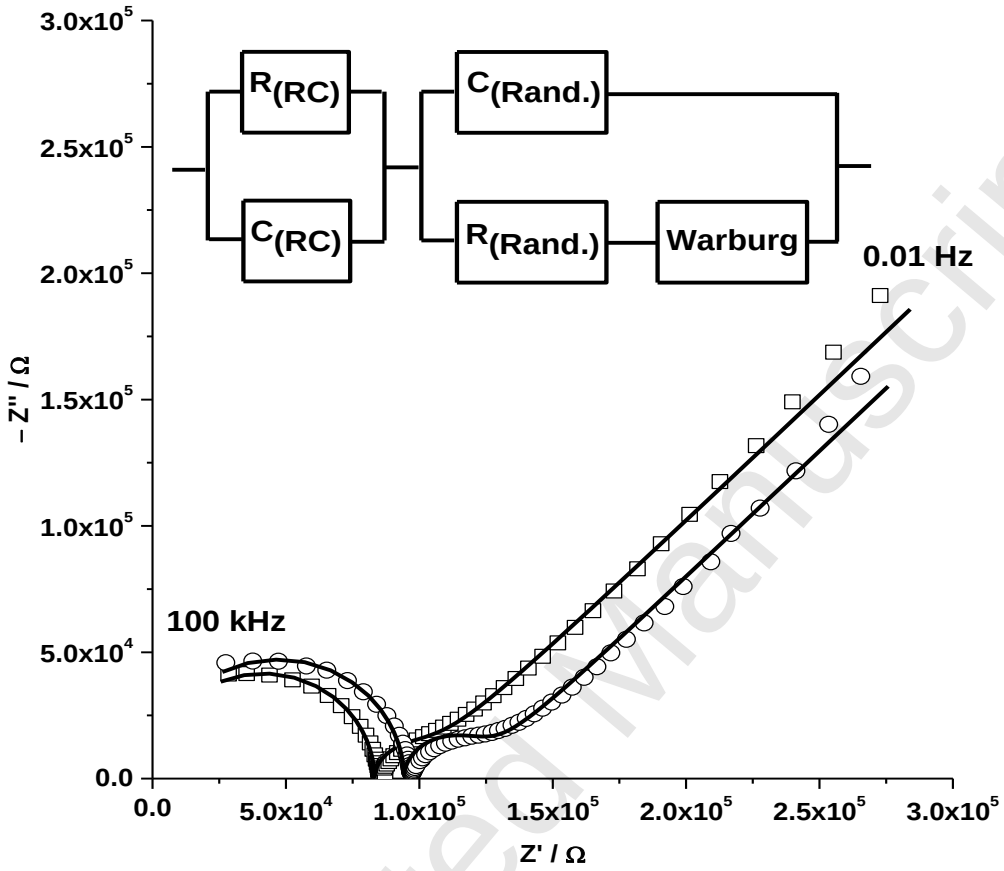

Fig. 4. Nyquist plots representing selected typical impedance response of the studied solid-contact ISEs together with the fit (solid lines), where □ is a Pb^{2+}-ISE conditioned in $Pb(NO_3)_2$ (**IV**) and ○ is a Pb^{2+}-ISE conditioned in PBS, BSA and $Pb(NO_3)_2$ (**V**). The numerals (**IV**) and (**V**) refer to **Scheme 1**. Insert: equivalent circuit used for fitting.

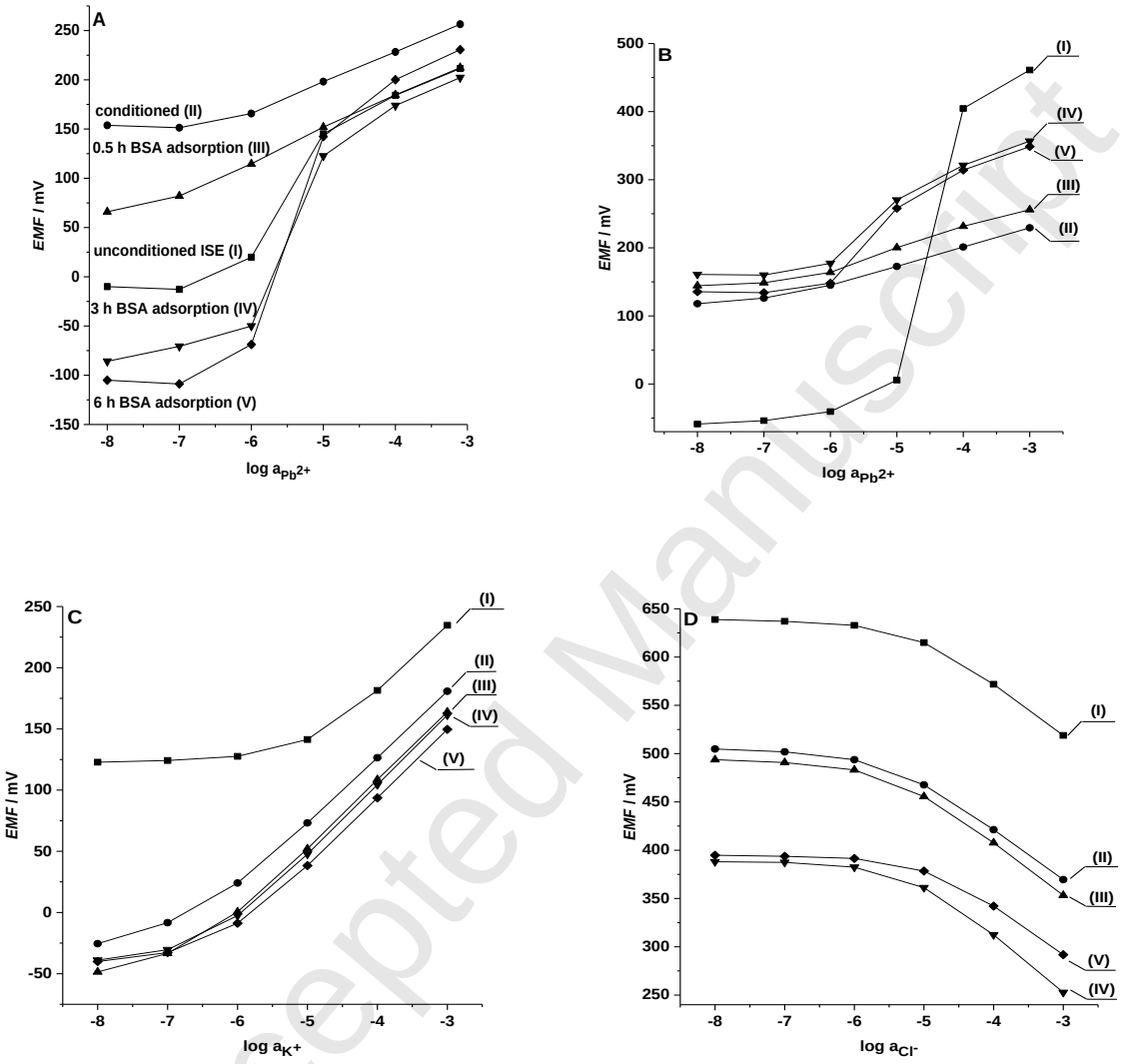

Fig. 5. Potentiometric response of Pb^{2+}-ISEs (A and B), K^{+}-ISEs (C) and Cl^{-}-ISEs (D) in primary ion solutions from 10^{-3} to 10^{-8} mol dm^{-3} after various conditioning procedures, namely: (I) unconditioned, (II) 24 h conditioning in 10^{-3} mol dm^{-3} of primary ion, (III) 0.5 h conditioning in solution 1 or 2, (IV) 3 h conditioning in solution 1 or 2, (V) 6 h conditioning in solution 1 or 2, where solution 1 was 10^{-2} mol dm^{-3} PBS with 1 mg ml^{-1} BSA (A, C, D) and solution 2 was 10^{-2} mol dm^{-3} PBS (B).

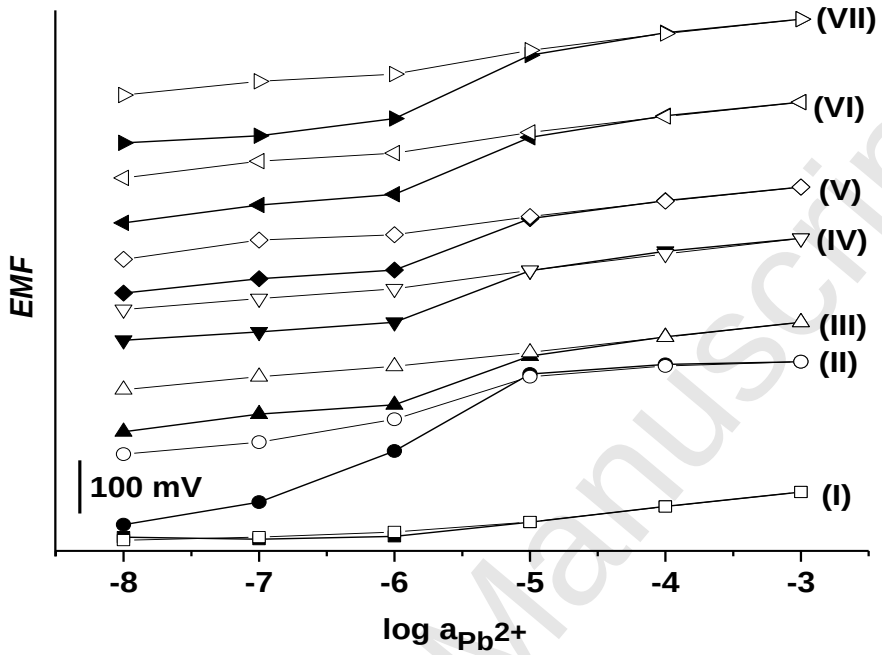

Fig. 6. Potentiometric response of Pb^{2+}-ISEs measured for increasing Pb^{2+} concentrations (filled symbols), followed by decreasing Pb^{2+} concentrations (open symbols) after application of different conditioning solutions: conditioned in 10^{-3} mol dm^{-3} Pb^{2+} for 24 h (I) followed by conditioning in 0.01 mol dm^{-3} PBS for 3 h (II) and followed by conditioning for 3 h in 0.01 mol dm^{-3} PBS containing 0.1 (III), 0.25 (IV), 0.5 (V), 1 (VI) and 2 (VII) mg ml^{-1} BSA.

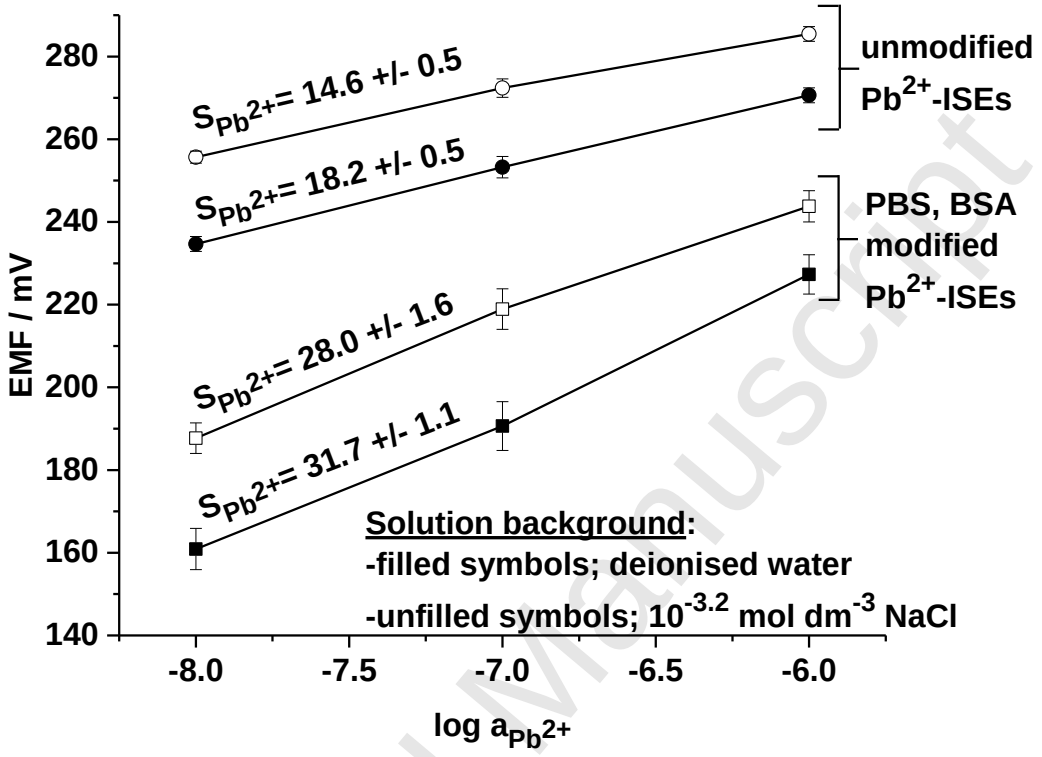

Fig. 7. Potentiometric response of unmodified (circles) and BSA and PBS modified (squares) Pb^{2+}-ISEs in standard solutions of $Pb(NO_3)_2$ measured between10^{-8} and 10^{-6} mol dm^{-3} Pb^{2+} with various standard solution background, namely deionized water (filled symbols) and $10^{-3.2}$ mol dm^{-3} NaCl (unfilled symbols).